Iranian Journal of Veterinary Science and Technology
Vol. 2, No. 1, 2010, 25-32

IJVST

Screening of Chloramphenicol Residues in Broiler Chickens Slaughtered in an Industrial Poultry Abattoir in Mashhad, Iran

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Received: February 20, 2010 Accepted: June 25, 2010

Abstract

The use of chloramphenicol is prohibited in food producing animals due to its harmful and even potentially fatal side effects in human. In order to screen broiler carcasses for the drug residues, 31 broiler chickens from different farms were sampled. The samples from kidneys were homogenized, extracted using ethyl acetate and dried under N_2 flow. The samples were then assayed using enzyme-linked immunosorbent assay (ELISA). In the next phase, the concentration of chloramphenicol in the kidney, liver and thigh muscle of 13 positive chickens was compared following extraction and ELISA as mentioned. More than half of the samples (54.8%) showed detectable concentrations of chloramphenicol. The highest concentrations of the drug were in the kidney and liver. According to the current research, there seems be a public health threat due to the illegal use of chloramphenicol in broiler farms and that kidney samples can be used for screening tests.

Keywords: chloramphenicol, drug residues, broiler chicken, poultry

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Introduction

Chloramphenicol has been widely used both in medical and veterinary practice, due to its broad spectrum antimicrobial activity as well as its remarkable penetration into the tissues. Although reasonably safe in domestic animals, however, it is known to exert several side effects in humans such as allergic reactions, gastrointestinal disorders, dose-dependent bone marrow depression and grey syndrome in newborns. The most serious and potentially lethal effect of chloramphenicol is aplastic anaemia (Holt *et al.*, 1993; Page, 1991; Papich and Riviere, 2001; Wareham and Wilson, 2002). This rare unpredictable disorder is not dose dependent and can occur with even extremely low ophthalmologic doses (Blogg, 1991; Lam *et al.*, 2002). The possible fatal effect in human has led to restriction on the veterinary uses of chloramphenicol. Food and Drug Administration (FDA) regulations have banned chloramphenicol from use in food animals. Therefore, there are no withdrawal times and no safe residue levels in meat, eggs, milk and other food with animal origin (FDA, 1997). Despite legal prohibition on use in domestic animals in Iran, there is evidence suggesting chloramphenicol may be widely used in poultry farms. This research was a preliminary study on residues of this antibiotic in certain tissues of broiler chickens that were slaughtered in an industrial abattoir in Mashhad.

Materials and methods

Animals

The study was performed on the broiler chickens that were slaughtered in an industrial abattoir in Mashhad. Thirty one samples out of 78500 chickens were randomly collected from 31 farms. Sampling intervals were adjusted to avoid repeated sampling from a single farm. Samples from liver, kidney and thigh muscle were transferred ice-cold to the laboratory and kept at -30°C.

Sample preparation

The samples were thawed at 4°C and homogenized using a mortar and a pestle for 20 min. The homogenized samples were then processed according to the protocol suggested by the kit supplier (Tecna Srl, Italy). In summary, 3g of each homogenized sample was mixed with 6ml ethyl acetate using a rotary shaker for 30min. The samples were then centrifuged at 2000×g for 10min. The supernatant was collected and dried under N_2 flow at 50°C. The pellet was dissolved in 1.5ml iso-octane:chloroform (2:3, V:V) and 0.75 ml sample extraction buffer (PBST: $Na_2HPO_4 2H_2O$, 0.96g; KH_2PO_4, 0.17g; NaCl, 9g; Tween 20, 0.5ml; distilled water lL), and the whole was mixed for 30 minutes and centrifuged (10min, 2000×g). The samples were then stored at -20°C till assayed.

ELISA procedure

A commercial enzyme linked immunosorbent assay (ELISA) kit (Tecna Srl, Italy) was used. The kit had a specificity of 100% for chloramphenicol and 70% for chloramphenicol glucuronide but showed only 0.01% cross reactivity with thiamphenicol, florphenicol and florphenicolamine according to the supplier. The assay was performed according to the procedure suggested by the kit. All standards and samples were run in duplicates. Fifty microliters of standard/sample was added to each well of microtiter plate coated with an anti-chloramphenicol antibody. Following addition of 100µl enzyme conjugate and 100µl antibody to each well, the microtiter plate was

incubated at 2-8°C for 2 hours. After three washes with washing solution, 150µl developing solution (containing chromogen and citrate buffer) was added to the wells. The microtiter plate was then covered to avoid direct light and incubated at room temperature for 30 minutes. The reaction was terminated with 50µl of stop solution (containing sulfuric acid 2M), absorbance was then measured at 450nm using a microplate reader. The concentrations of chloramphenicol in the samples were calculated according to the percentage of their mean absorbance divided by the absorbance of the maximum binding (B/B_O%) using the standard curve. The values were then multiplied by the dilution factor (0.25) as suggested by the kit manual.

Experimental protocol

The study was performed at two steps. At the first stage, only the kidney samples from 31 chickens were assayed for possible chloramphenicol residues. At the second stage, 13 chickens with the highest concentrations of chloramphenicol in their kidneys from the first stage were selected, and the tissue levels of chloramphenicol in their kidneys, livers and thigh muscles were measured.

Statistical analysis

The standard curve (Fig 1) was plotted using linear regression, and the amounts of chloramphenicol in the samples were interpolated using GraphPad Prism (GrapPad Software Inc, USA) software. The amounts of drug residues in different tissues (kidney, liver and thigh muscle) were compared using ANOVA for repeated measures followed by Tukey's multiple comparison test. Unless otherwise stated, all data are expressed as mean ± SEM; and statistical difference with $p<0.05$ is considered as significant.

Results

The standard curve (Fig 1) had a slope of -95.05± 4.33, with an r^2 value of 0.98 ($p<0.0001$). The detection limit of the assay was 0.05ng/ml and only values above this concentration were considered as positive. The coefficient of variation was 19.89% (0.5ng/ml, n=6). In the first stage of the experiment, 14 samples (45.2%), out of a total of 31 samples, showed values less than 12.5 pg/g immunoreactivity and were considered as negative (Fig 2). The positive samples showed different levels of immunoreactive chloramphenicol, 35.5% showed levels between 12.5-250pg/g, whereas 19.3% demonstrated levels above 250pg/g kidney tissue.

In the second stage of the experiment, tissue levels of immunoreactive chloramphenicol in kidney, liver and thigh muscle were compared using a separate ELISA kit (Fig 3). The highest concentrations of immunoreactive chloramphenicol were detected in kidney and liver tissues. The levels of chloramphenicol residue in thigh muscle samples were significantly lower compared to those in the kidney.

Discussion

Chloramphenicol induced bone marrow aplasia is not dose dependent, and even extremely low ophthalmologic doses are known to cause aplastic anemia in human. This, together with other toxic and carcinogenic effects of chloramphenicol, has caused particular concern for the public health (Allen, 1985; Papich and Riviere, 2001). The number of animals receiving the drug in industrial broiler farms is huge, and their tissue residue of chloramphenicol is particularly important in this regard. Therefore, many countries have prohibited the use of chloramphenicol in food producing animals. In addition to FDA, a Joint Food and Agriculture

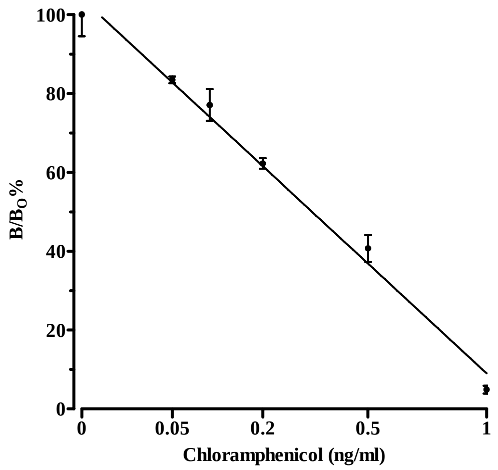


Figure 1- Standard curve of chloramphenicol using ELISA (r^2 =0.98; P<0.0001; n=2).

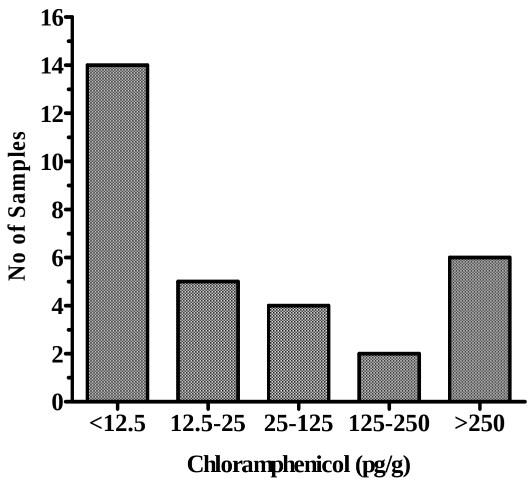


Figure 2- The concentration of immuno-reactive chloramphenicol in the kidney of the broiler chickens. Values less than 12.5pg/g were below the detection limit of the assay and were considered as negative.

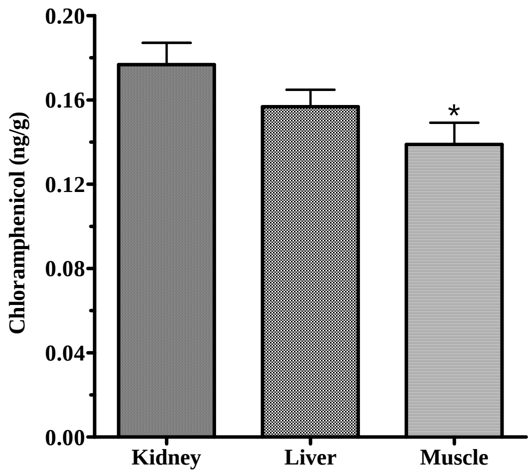


Figure 3- The residue levels of chloramphenicol in the homogenates of kidney, liver and thigh muscle of chloramphenicol positive chickens. The asterisk indicates statistical significance (p<0.05) compared to the values in the kidney. All other comparisons are non-significant (ANOVA for repeated measures, Tukey's multiple comparison test; n = 13).

Organisation/World Health Organisation (FAO/WHO) Expert Committee on Food Additives (JECFA) has proclaimed that chloramphenicol residues in human food supply are unacceptable (World Health Organization, 1994). Consistently, the presence of chloramphenicol in food has been illegal and unacceptable in European Community (EC) since 1994 (European Community Regulations, 1994).

Appropriate decisions are made by some countries to test suspicious food products of animal origin in order to ensure the absence of chloramphenicol residues (Cerkvenik, 2002; Impens *et al.*, 2003). Various methods such as HPLC (Hummert *et al.*, 1995; Posyniak *et al.*, 2003), GC (Gude *et al.*, 1995; Pfenning *et al.*, 1998), GC-MS (Impens *et al.*, 2003; Shen and Jiang, 2005), HPLC-MS (Impens *et al.*, 2003; Takino *et al.*, 2003), radio-immunoassay (Arnold and Somogyi, 1985) and ELISA (Gaudin *et al.*, 2004; Impens *et al.*, 2003; Scortichini *et al.*, 2005) have been developed and introduced for detection of chloramphenicol residues in food animals. Most of these methods have detection limits below 0.1ng/g which is required for monitoring chloramphenicol residues in animals. The ELISA used in this study, however, had a detection limit of 0.012ng/g. Even lower detection limits may be achieved by some modifications in the assay (Zhang *et*

al., 2006). In addition to high sensitivity, ELISA is fast and cheep, and hence, it seems ideal for screening tests. It should be noted, though, that there may be false positive results with ELISA, and that suspected results should be confirmed with chromatographic mass spectrometric detection (Impens *et al.*, 2003).

There are some reports of chloramphenicol analysis and monitoring in food of animal origin in other countries. In Slovenia, for instance, the screening of chloramphenicol residues for statutory purpose started in 1991 (Cerkvenik, 2002). In Belgium, ELISA has been used for screening tissue residuals of chloramphenicol in shrimps (Impens *et al.*, 2003). In UK, demand for chloramphenicol assays has dropped dramatically because newer less toxic antibiotic are available, and also since HPLC has replaced ELISA due to financial reasons (White, 2000). In the Islamic Republic of Iran, there is one report of study on chloramphenicol residues in cow milk (Moshafi and Mortazavi, 1991). This research was mainly aimed to determine the necessary withdrawal time following intra-mammary or i.m. injection of the drug. However, there is no report of screening the residuals of this drug in broilers. This study was aimed to screen the risk of chloramphenicol contamination in slaughtered broilers in Mashhad. The high percentage of suspicious samples in this study is alarming for the public health.

In order to identify the ideal tissue for screening studies, the concentrations of chloramphenicol were compared in different tissues (kidney, liver and thigh muscle) of some positive samples. The highest concentrations of the immunoreactive drug were detected in the kidney and liver respectively. It is noteworthy that chloramphenicol is mainly metabolized in liver via conjugation with glucuronic acid, and up to 90% of the drug and its metabolites are excreted in urine (Papich and Riviere, 2001; World Health Organization, 1994). It is, therefore, conceivable to detect the highest concentrations of the drug/metabolites in kidney and liver.

In conclusion, the current study suggests a high risk of illegal use of chloramphenicol in broiler farms, and that strict measures should be taken in this regard. The kidney tissue may be used for screening tests for tissue residuals of chloramphenicol in broilers.

Acknowledgment

We are grateful to the School of Veterinary Medicine, Ferdowsi University of Mashhad for funding this research (Grant no: 9.3865). We also thank Mrs S. Khajenasiri for her technical assistance.

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Iranian Journal of Veterinary Science and Technology
Vol. 2, No. 1, 2010, 25-32

IJVST

بررسی بقایای کلرامفنیکل در نیمچه‌های گوشتی کشتار شده در یک کشتارگاه صنعتی طیور در مشهد، ایران

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دریافت مقاله: ۸۸/۱۲/۱ پذیرش نهایی: ۸۹/۴/۴

چکیده

استفاده از کلرامفنیکل در حیوانات حلال گوشت، به خاطر عوارض جانبی و حتی احتمال خطر مرگ که در انسان به همراه دارد، ممنوع می‌باشد. جهت بررسی آلودگی لاشه نیمچه‌های گوشتی به بقایای این دارو، از ۳۱ قطعه مرغ که از مرغداری‌های متفاوتی بودند، نمونه گیری به عمل آمد. نمونه‌های مربوط به کلیه، همگن شده و پس از استخراج توسط اتیل استات، با استفاده از گاز ازت خشک شدند. سپس نمونه‌ها توسط آزمایش الایزا مورد سنجش قرار گرفتند. در مرحله بعد، غلظت کلرامفنیکل در کلیه، کبد و عضله ران ۱۳ قطعه از لاشه‌هایی که طی مرحله اول آلوده تشخیص داده شده بودند، بر اساس توضیحات فوق، پس از استخراج، توسط الایزا اندازه گیری شد. بیش از نیمی از نمونه‌ها (۵۴/۸٪)، غلظت‌های قابل ردیابی کلرامفنیکل نشان می‌دادند. بالاترین غلظت دارو در کبد و کلیه مشاهده گردید. بر اساس این پژوهش به نظر می‌رسد به خاطر مصرف غیر قانونی کلرامفنیکل در مرغداری‌های گوشتی، بهداشت عمومی در معرض خطر است و در این رابطه می‌توان از نمونه‌های کلیه جهت آزمون های غربالگری استفاده نمود.

واژگان کلیدی: کلرامفنیکل، بقایای دارویی، نیمچه‌های گوشتی، طیور